



## Short Communication

# Isolation of viable *Neospora caninum* from brains of wild gray wolves (*Canis lupus*)



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## ABSTRACT

*Neospora caninum* is a common cause of abortion in cattle worldwide. Canids, including the dog and the dingo (*Canis familiaris*), the coyote (*Canis latrans*), and the gray wolf (*Canis lupus*) are its definitive hosts that can excrete environmentally resistant oocysts in the environment, but also can act as intermediate hosts, harboring tissue stages of the parasite. In an attempt to isolate viable *N. caninum* from tissues of naturally infected wolves, brain and heart tissue from 109 wolves from Minnesota were bioassayed in mice. Viable *N. caninum* (NcWolfMn1, NcWolfMn2) was isolated from the brains of two wolves by bioassays in interferon gamma gene knockout mice. DNA obtained from culture-derived *N. caninum* tachyzoites of the two isolates were analyzed by *N. caninum*-specific Nc5 polymerase chain reaction and confirmed diagnosis. This is the first report of isolation of *N. caninum* from tissues of any wild canid host.

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## 1. Introduction

The protozoan *Neospora caninum* infects many species of warm blooded animals and is a major cause of bovine abortion worldwide (Dubey et al., 2007). Its life cycle involves canids as definitive hosts and many other species as intermediate hosts. The domestic dog and the Australian dingo (*Canis familiaris*), gray wolf (*Canis lupus*), the coyote (*Canis latrans*) are its definitive hosts and they shed environmentally resistant oocysts in their feces (McAllister et al., 1998; Basso et al., 2001; Gondim et al., 2004a; King et al., 2010; Dubey et al., 2011).

Although antibodies to *N. caninum* have been found in many hosts, including mammals and birds, viable parasites are rarely isolated (Dubey et al., 2007; Dubey and Schares,

2011). Whether canids, other than the dog, can act as intermediate hosts is unknown. Little is known of the genetic diversity and biological characteristics of *N. caninum* isolates because it is difficult to isolate viable parasites from naturally-infected hosts, specially wildlife. The purpose of this study was to isolate viable *N. caninum* from wolf brains in order to improve our understanding of the epidemiology of neosporosis, particularly the sylvatic life cycle.

As part of investigation on examination of wolves for different pathogens (Butler et al., 2011), sera, tissues, and feces of wolves from Minnesota were examined for evidence of *N. caninum*, and *Toxoplasma gondii* infections. Results of isolation of *N. caninum* oocysts in wolf feces (Dubey et al., 2011), and results of *T. gondii* investigation on these wolves were reported previously (Dubey et al., 2013a). Results of *Neospora* serology and other pathogens will be described by the Minnesota Department of Natural Resources (MNDNR) (in preparation). This paper concerns only regarding isolation of *N. caninum* from wolf tissues.

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## 2. Materials and methods

### 2.1. Naturally infected wolves

From May 2010 through July 2012, the MNDNR collected biological samples from depredating wolves through a contract with the United States Department of Agriculture (USDA)'s Animal and Plant Health Inspection Service Wildlife Services. Additional samples were obtained from vehicle-killed wolves, as well as from researchers within the MNDNR, the United States Geological Survey, Camp Ripley military base, and tribal authorities that live-capture and radio-collared wolves. For the present study, samples were collected post-mortem.

Blood was collected from a jugular vein whenever possible (cephalic vein or saphenous vein are also options). For euthanized wolves, blood was collected from the site of a bullet wound, heart, or from the chest cavity as soon after death as possible; the wolves were shot by personnel trained in the safe use of fire arms. Serum was recovered by centrifugation of blood samples at 5000 g for 10 min. Heart and brain samples were also collected from euthanized wolves when possible. Samples were submitted to the Animal Parasitic Diseases Laboratory (APDL), USDA, Beltsville, Maryland for protozoal examination, including *T. gondii*, *N. caninum*, and *Sarcocystis neurona* that are related coccidioids ([Dubey et al., 2013a](#)). Not all samples were available from each wolf.

### 2.2. Serological examination

*N. caninum* agglutination test (NAT) was used to detect antibodies to *N. caninum*. The NAT was performed with mouse-derived whole tachyzoites antigen as described by [Romand et al. \(1998\)](#). Antibodies to *T. gondii* were assayed by the modified agglutination test as described by [Dubey and Desmonts \(1987\)](#).

### 2.3. Bioassay in mice

Tissues (brains alone of 12, hearts alone of 20, brain and heart compared for 26, and brain and heart pooled for 51) of 109 wolves were bioassayed in mice using trypsin or pepsin digestion as described previously for *T. gondii* investigation ([Dubey et al., 2013a](#)). Bioassay procedures for *N. caninum* and *T. gondii* are essentially similar. The selection of tissues to be bioassayed was based on *T. gondii* serologic examination of wolves ([Dubey et al., 2013a](#)). Sera were retrospectively assayed for *N. caninum* antibodies. The number and kind of mice used, tissues (brain, heart, or both) bioassayed, and method of bioassay varied during the course of investigation depending on the availability of mice when the samples arrived, degree of autolysis of tissues, and the serological status of the wolves. Two kinds of mice were used for bioassay. Outbred albino Swiss Webster (SW) mice were obtained from the National Cancer Institute, Bethesda, Maryland. The interferon gamma (C57-black-derived) gene knockout (KO) were obtained from the Jackson Laboratories (Bar Harbor, Maine) or bred in-house from the stock from Jackson. The KO mice were used to

facilitate isolation of *N. caninum* because outbred mice are not a good host for *N. caninum*.

For digestion of hearts, the myocardial samples (30 g) were homogenized in 0.85% aqueous NaCl (saline), digested in pepsin, centrifuged, suspended in saline and inoculated in to SW and KO mice as described previously ([Dubey, 2010](#)).

For trypsin digestion, brains (30 g) were homogenized in 5 volumes aqueous 0.85% NaCl solution (saline) in a blender. To this brain homogenate an equal volume of 0.5% trypsin in saline was added so that the final concentration of trypsin in the homogenate was 0.25%. The homogenate was incubated at 37 °C in a water bath for 1 h. The digest was passed through two layers of gauze, centrifuged for 10 min at 1400 g, and the supernatant was discarded. The sediment was suspended in saline and the process was repeated twice to remove trypsin. The final sediment was suspended in antibiotic saline (1000 units penicillin, 100 micrograms streptomycin per ml saline), and aliquots were inoculated subcutaneously in to SW mice and KO mice. Mice that died or were euthanized when ill were examined for protozoal infections. Survivors were bled two months later and their sera were examined for antibodies to *T. gondii* and *N. caninum* using respective agglutination tests at serum dilution of 1:25.

### 2.4. Necropsy and immunohistological examination

Samples of all major organs of infected mice were fixed in 10% buffered formalin. Paraffin-embedded tissues were sectioned at 5 µm and examined after staining with hematoxylin and eosin (H and E). Immunohistochemistry was performed on paraffin-embedded sections at APDL using reagents and methods described previously by [Lindsay and Dubey \(1989\)](#).

### 2.5. In vitro cultivation

Lung tissue from KO mouse with demonstrable protozoal tachyzoites was homogenized in RPMI-1640 medium supplemented with L-glutamine, and seeded on to M617 bovine monocyte culture or CV1 cells ([Dubey, 2010](#)). The cell cultures were observed microscopically for growth of *N. caninum* for three months.

### 2.6. DNA extraction and polymerase chain reaction (PCR) amplification

DNA from cell cultures inoculated with *N. caninum* isolated from mouse lung were extracted using the QIAamp Mini DNA kit (QIAGEN Inc., Valencia, CA, USA), and subjected to PCR amplification of the Nc5 gene sequence ([Kaufmann et al., 1996; Müller et al., 1996; Liddell et al., 1999](#)) using PrimeStar Max DNA polymerase and the PrimeStar amplification kit (Clontech Laboratories, Inc., Mountain View, CA, USA). Reaction conditions consisted of an initial denaturation at 94 °C, 3 min, followed by 35 cycles 94 °C, 30 s, 63 °C, 30 s, 72 °C, 1 min, and final extension at 72 °C, 5 min. PCR products were analyzed by acrylamide gel electrophoresis followed by EtBr staining, and visualization and capture on a Gel Logic 200 Imaging System (Eastman

Kodak, Hemel Hempstead, UK). The Nc5 target amplification product was isolated using a QIAquick PCR purification kit (QIAGEN Inc.), inserted into pGEM-T easy cloning vector (Novagen, San Diego, CA, USA), and introduced into *Escherichia coli* DH5 using standard procedures (Hanahan, 1983), followed by colony PCR analysis (Güssow and Clackson, 1989). At least three separate PCR amplifications were conducted for each DNA sample, and a minimum of three recombinant clones were evaluated for each amplification product. Colony amplification reactions exhibiting the expected size product by polyacrylamide gel electrophoresis were then subjected to sequence analysis using M13 forward and reverse primers, and a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA). DNA sequences termed NcWolf1–NcWolf5 were verified as Nc5 by BLAST-N analysis of sequences deposited in the GenBank database (Accession Nos. KF649844 to KF649848).

### 2.7. Animal ethics

Wolves prey on livestock and there is continued debate concerning curtailing the increasing wolf population in the western United States. Some of the wolves were causing depredation problems and therefore trapped and shot by USDA-Wildlife Services.

All wolves were handled in accordance to approved protocols and laboratory experiments were also conducted using approved protocols.

## 3. Results

Viable *N. caninum* was isolated from trypsin digested brains of two male wolf pups killed on September 11, 2010. They were born most likely in late April–early May; thus about 4–5 months old. They were also killed at the same location, 46.78852 northing, –92.62871 easting (decimal degrees) or UTM 528339 easting, 5181730 northing in St. Louis County, MN. There is a very high probability that these 2 wolves were from the same pack, and therefore littermates. Details of both isolates are as follows:

### 3.1. Wolf 1 (W-09-11-10WJP1)

Trypsin digest of brain of this wolf was inoculated into two SW and three KO mice. The SW mice remained asymptomatic and neither antibodies nor tissue cysts of *N. caninum* were found when mice were killed 50 days p.i. All three KO mice inoculated with brain homogenate became ill; KO #1 died day 30 p.i. KO #2 was euthanized day 30 p.i. and KO #3 was euthanized day 44 p.i. Tachyzoites were found in lung smears made from lungs of all three KO mice. Tissues of KO mouse #1 were discarded because of autolysis. Complete necropsy was performed on KO #2. Grossly, its heart was hypertrophied, lungs were edematous and focally consolidated, and liver was gritty. Its tissues were fixed for histological examination, and its lung homogenate was inoculated on to cell cultures. Immunohistochemically, *N. caninum* tachyzoites were found in sections of adrenal, lungs, liver, and brain. *Neospora* tachyzoites were found in cultures of M617 bovine monocyte

culture flask; 90% of host cells were destroyed by 10 day p.i. The medium containing free tachyzoites was centrifuged and the sediment saved for PCR. The flask was scraped and the tachyzoites were cryopreserved and the strain was designated NcWolfMn1. The tissues of KO mouse #3 were discarded after finding tachyzoites in lung smears.

### 3.2. Wolf 2 (W-09-11-10WJP2)

Trypsin digest of brain of this wolf was also inoculated into two SW and three KO mice. Both the SW mice and two of the three KO mice remained asymptomatic and neither antibodies nor tissue cysts of *N. caninum* were found when mice were killed 75 days p.i. The third KO mouse (#4) died day 29 p.i.; protozoal tachyzoites were found in its lung smear. Lung homogenate of KO #4 was inoculated on to cell culture and also in three SW and two KO mice. *Neospora caninum* tachyzoites were propagated successfully in CV1 cells and the strain was designated NcWolfMn2 and culture was cryopreserved. The three SW mice remained asymptomatic; antibodies to *N. caninum* were detected in 1:25 dilution of their sera but no protozoal organisms were found in their brain when mice were killed day 69 p.i. The two KO mice inoculated with infected lung homogenate were euthanized day 15 p.i.; tachyzoites were found in smears of their lungs.

### 3.3. DNA characterization

*N. caninum*-specific Nc5 polymerase chain reaction (PCR) of DNA from both isolates corroborated clinical and serological diagnosis of *N. caninum* infection. DNA sequencing of over 50 individual clones from either isolate, and BLAST-N analysis of GenBank database revealed greater than 96% identity to deposited Nc5 sequences. Alignment of these sequences using CLUSTAL revealed 5 distinct Nc5 sequences with variation at 13 nucleotides, representing greater than 98% similarity in the sequences (GenBank accession nos. KF649844 to KF649848). The SNPs were equally distributed in Nc5 sequences from both isolates.

## 4. Discussion

In the present study, *N. caninum* was isolated from only two wolves; in both instances, the parasite was isolated from the brain and not heart which is in keeping with the biology of the parasite. *N. caninum* encysts more commonly in the CNS than the heart (Dubey et al., 2007). Another drawback of bioassay used is that most of the wolves bioassayed were seropositive for *T. gondii*; *T. gondii* usually grows faster in mice and cell culture than *N. caninum*, and thus some isolates of *N. caninum* could have been missed. Fortunately, there was no co-infection with *T. gondii* in the two isolates reported here.

The sources of infection for *N. caninum* infection in wolves in Minnesota or elsewhere are unknown; the white-tailed deer and cattle are commonly infected with *N. caninum* in the USA (Gondim et al., 2004b). *N. caninum* antibodies were found in 80 (16.5%) of 485 white tailed deer from the same region of Minnesota as the wolves, and viable *N. caninum* was isolated from brains of two deer

fetuses in Minnesota (Dubey et al., 2009, 2013b). Although neosporosis is a common cause of abortion in cattle worldwide there is no published report of *N. caninum* infection in cattle or other hosts in Minnesota. Wolves prey on livestock and there is continued debate concerning curtailing the increasing wolf population in the western United States. The wolves from whom *N. caninum* was isolated were part of a pack that was causing depredation problems and therefore trapped and shot by USDA-Wildlife Services.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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